A CYTOCHEMICAL LOCALIZATION OF REDUCTIVE SITES IN A GRAM-NEGATIVE BACTERIUM

Tellurite Reduction in *Proteus vulgaris*

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ABSTRACT

In order to obtain information on the exact location of the respiratory enzyme chain in Gram-negative bacteria, an electron microscopic study was made of the sites of reducing activity of cells that had, in the living state, incorporated tellurite. In the test object *Proteus vulgaris*, the reduced tellurite was found to be deposited in bodies contiguous with the plasma membrane but different in structure from those described in the Gram-positive *Bacillus subtilis* (2). In fact, the bodies proved to consist of a conglomerate of elements which contained the strongly electron-scattering reduced tellurite and a delicately granular "matrix." A limiting membrane was not observed around these complexes. In serial sections details of the complexes are illustrated. Reduced tellurite was not deposited in the plasma membrane to any important degree. Since no other sites of deposition of the reduced product were revealed, it is assumed that the complexes represent the mitochondrial equivalents in the investigated organism. In addition, the bodies might function as the basal granules of the flagella.

INTRODUCTION

By the use of the strongly electron-scattering reduced tellurite as an indicator in localizing by electron microscopy the respiratory enzyme chain in *Bacillus subtilis*, a reductive system was found (2) to be contained in the membranes of previously described "organelles" (chondrioids). In addition, at the periphery of the cells slender rod-like structures were observed, opacified by reduced tellurite. In the preceding paper, types of bacteria, in which typical membranous organelles have been detected, were surveyed, and all these organisms turned out to be Gram-positive.

In the Gram-negative bacteria studied so far in our laboratory, *i.e.*, *Proteus vulgaris*, *Hemophilus influenzae*, *Spirillum serpens*, *Rhodospirillum rubrum*, and *Azotobacter vinelandii*, we have observed neither the vesiculo-tubular nor the concentric lamellar organelles. There are plenty of membranes in *Rhodospirillum rubrum* and in *Azotobacter vinelandii*, but these are of a different character (1). Nor have we found in the literature any convincing evidence of the occurrence in Gram-negative bacteria of membranous bodies comparable to those in Gram-positive organisms. In *Escherichia coli*, membrane systems are, as a rule, not observed (3–5), although simple membranous formations have been found to occur under unfavourable conditions (6, 5); for *Spirillum serpens*, Murray (7) and VanderWinkel and Murray (5) described invaginations of the plasma membrane but admitted that generally these profiles are simple and only rarely assume the concentric membrane con-

figurations. Since equivalents of mitochondria should be present in sufficient number in all aerobic cells, it may be expected that in Gram-negative bacteria the situation of the respiratory chain is different from that in Gram-positive ones. The present investigation has been undertaken to locate the sites of tellurite reduction in Gram-negative bacteria, using *Proteus vulgaris* as a test object.

Studies on the reduction of tellurite by *Proteus* have been published by Nermut (8, 9). In our methods, use has been made of some of his results.

MATERIAL AND METHODS

Proteus vulgaris, the strain from which Mrs. E. Klieneberger-Nobel derived her stable L form L9, was grown in heart infusion broth (Difco) at a pH of 6.6 (8). To the agitated cultures in the early logarithmic phase (4 hours), potassium tellurite (K_2TeO_3) was added to a final concentration of 0.05 per cent (8), and then the agitation was stopped (2). After an additional 4 hours of incubation at 37°C the cultures were centrifuged and the precipitate was treated as described for *Bacillus subtilis* (2). Cells used for embedding in Vestopal W were fixed according to Ryter and Kellenberger (3), using 1 per cent OsO_4, but in one experiment the OsO_4 was replaced by 6½ per cent glutaraldehyde, and in some samples in this experiment postfixation treatment with uranyl acetate was omitted.

Serial sections were made with the LKB Ultrotome.

Micrographs were taken with the Philips EM 200, using the double condenser lens system and an objective aperture of 25 μ.

OBSERVATIONS

As in our observations on *Bacillus subtilis* (2), the cultures of *Proteus vulgaris* grown with potassium tellurite in the medium gave, after centrifugation, a black precipitate with a clear supernatant. In shadowed whole cells, as in Figs. 1 and 2, the reduced tellurite can be discerned as opaque spots (at *T*). In Fig. 1, at *F*, the flagella can be traced to their possible origin in some of these dense structures. In Fig. 2 it is remarkable that even outside the cell, along the basal part of some of the flagella, a continuation of the deposits of the reduced tellurite is suggested (at *F*), which lends support to the supposition that the flagella have their bases in the structures underlying these accumulations of the reduced tellurite. Details of these opaque structures should be studied in thin sections cut through the outer part of the bacterium: Figs. 4 to 8 represent a series of five of such sections, starting just below the surface of the cell. In Figs. 9 to 13 another series of five sections illustrates details of the fine structure of a body containing the reduced product.

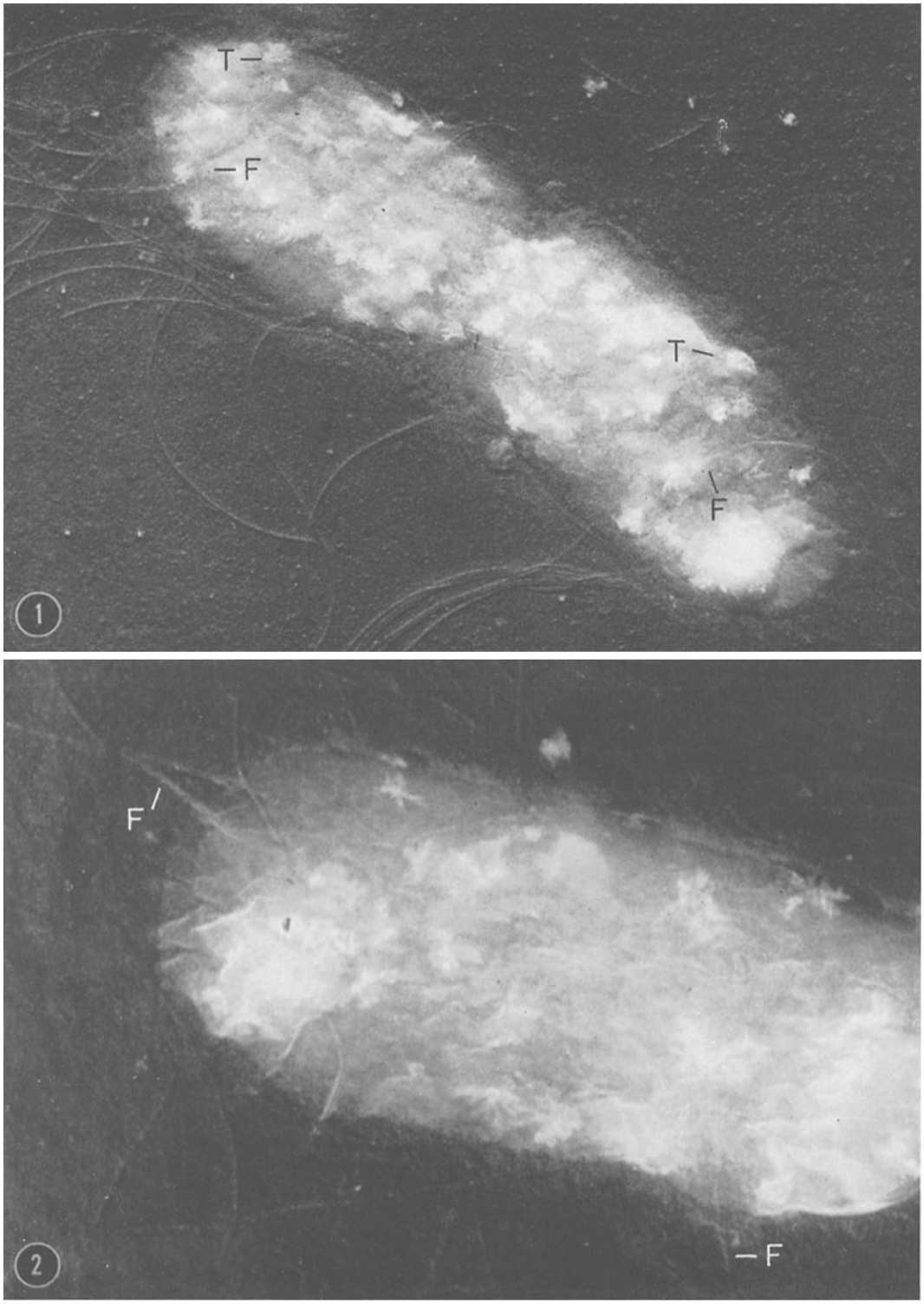
For comparison, a picture is included of an 8-hour-old cell of *Proteus vulgaris* grown under the same conditions as the cells to which potassium tellurite was administered in the culture medium (Fig. 3). In thin sections of such cells, little can be recognized of the structure which in the treated cells incorporated the reduced product. However, at *M* there is an area of very delicately granulated cytoplasm, comparable to what will be described

Abbreviations

T, structure containing reduced tellurite
F, flagellum
PM, triple-layered plasma membrane
R, rod-like structure containing reduced tellurite
G, rounded structure containing reduced tellurite
N, nucleoplasm
M, very finely granular cytoplasm

FIGURE 1 Flattened whole cell from a *Proteus* culture, grown for 4 hours with potassium tellurite in the medium, shadowed with platinum, and printed in reverse. The opaque, often somewhat rosette-like, structures are the bodies which incorporated the reduced tellurite (at *T*). At *F*, indications that the flagella could emerge from these bodies. × 38,000.

FIGURE 2 Same as Fig. 1. At *F*, a deposit along the basal part of the flagella even outside the cell suggests presence of reduced tellurite. × 57,000.


T—
—F
T—
F
1
F
—F
2

in association with the structures of increased density due to treatment with tellurite.

The equivalents of the opaque structures in Figs. 1 and 2 (at T) are, in the thin sections of Fig. 4, represented as opaque circumscribed areas (T_1 and T_2). In this figure, and in particular in the inset, two rods are seen to emerge from a tangle of thin dense filaments or sheets. The rod indicated at R has on its right side a delimitation of greater opacity than on its left side, and it shows a periodicity. The width of such rods is a little over 100 A, and the dense dots shaped as triangles (Figs. 7, 8, R) may well represent these rods in cross-section. The rods seem to penetrate the cytoplasm relatively deeply, but the centres of the cells always appear free of reduced tellurite. The seemingly entangled masses of filaments or sheets, tangentially cut in Fig. 4, are seen in cross-section to be situated in close relation to the plasma membrane (Figs. 7 and 8, at T). Occasionally, a dense rounded structure is seen near this mass: in Fig. 8 there are two of these globules (G_1 and G_2) to which extremely fine filaments or sheets are attached; at G_2 the latter have a zig-zag configuration (see insets). More strikingly than shown in Fig. 8 are delicate fibrils or membranes *ca.* 50 A in width which are attached to a rounded structure in Fig. 14.

If the flagella emerge from the conglomerates of dense structures, as suggested by Figs. 1 and 2, they should be looked for in the surface area of the cells. However, flagella were rarely noticed in these sections, and inside the cells indications of their presence are few. The circles *ca.* 120 A in diameter at F in Figs. 5 and 6 might perhaps represent flagella, in cross-section, leading towards the underlying conglomerates visible at T_5 and T_6 in Figs. 6 and 7.

The conglomerates of dense elements generally appear to contain an area of delicately granular cytoplasm, as seen also in the untreated cell in Fig. 3 (at M). Such areas (at M in Figs. 7 and 8) are demonstrated best in figs. 9 and 10. In the electron micrographs no membrane could be observed enclosing this "matrix," which seems in direct contact with the normally granular cytoplasm.

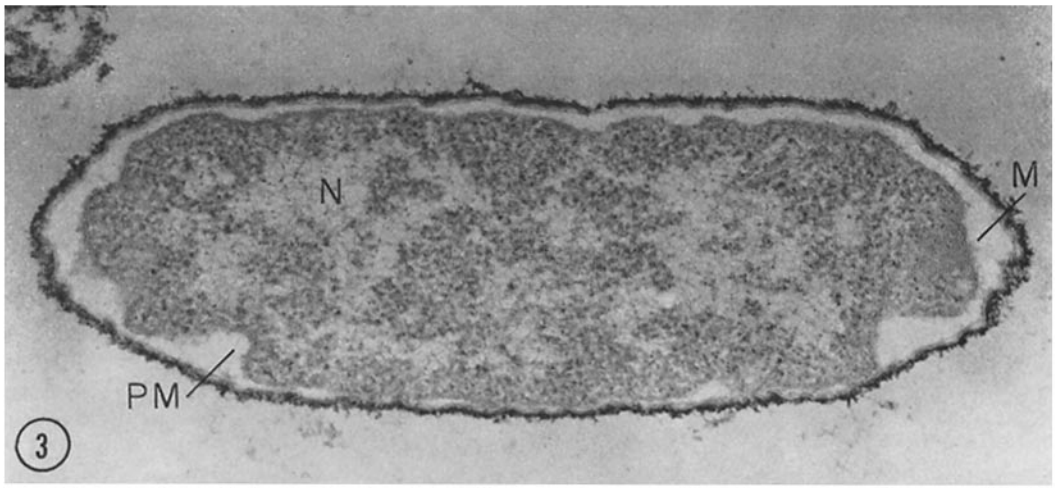
Cells fixed in glutaraldehyde most convincingly reveal the presence of the reduced tellurite deposits (Figs. 16 and 17) as compared with the non-treated cell (Fig. 15). Although in all three micrographs shown in Figs. 15 to 17 there is loss

FIGURE 3 Thin section of a cell from an 8-hour-old culture, grown in parallel with the cultures for the cells in Figs. 4 to 16, with omission of tellurite in the culture medium. The transparent region with an irregular network of filaments represents the nucleoplasm (N) meandering in the cytoplasm. At M, an area of very finely granular cytoplasm, similar to the "matrix" M in Figs. 7 to 10. In Gram-negative bacteria the cell wall does not fit the plasma membrane smoothly and completely, as it does in Gram-positive bacteria. In this cell the effect is exaggerated by shrinkage of the cytoplast, presumably during preparation. In this respect, the cell wall in this cell differs from that of well aerated cells in the early logarithmic phase, in that its outside surface is covered by dense material. At sufficiently high magnification, three dense sheets separated by lighter interspaces can be distinguished in the wall underneath this layer. × 84,000.

FIGS. 4 to 8 represent 5 serial sections of a tellurite-treated cell, beginning close to its surface. × 84,000.

FIGURE 4 The section shows the outer parts of two tangentially cut bodies, T_1 and T_2 which incorporated reduced tellurite. The inset, showing T_1 at higher magnification, demonstrates that the bodies have a mass of fibrils or sheets from which rods (R) protrude. Serial sections (Figs. 5 to 8) suggest that these rods are oriented inwards into the cytoplast. At R the fine structure of the rod is indicated: on one side it is bordered by a somewhat broader dense line than on its other side. Inset, × 158,000.

FIGURE 5 More centrally the tellurite-reducing bodies T_1 and T_2 of the former section show rods only. At T_3 is part of a conglomerate of structures opacified by the reduced tellurite, visible also in the next section. At F_1 and F_2 are cross-sections of hollow tubes *ca.* 120 A in diameter which perhaps are flagella.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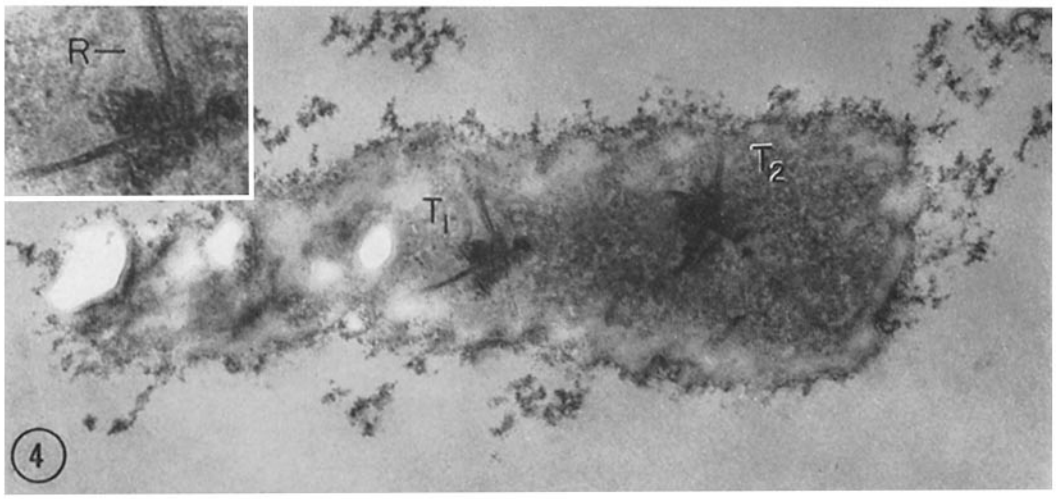
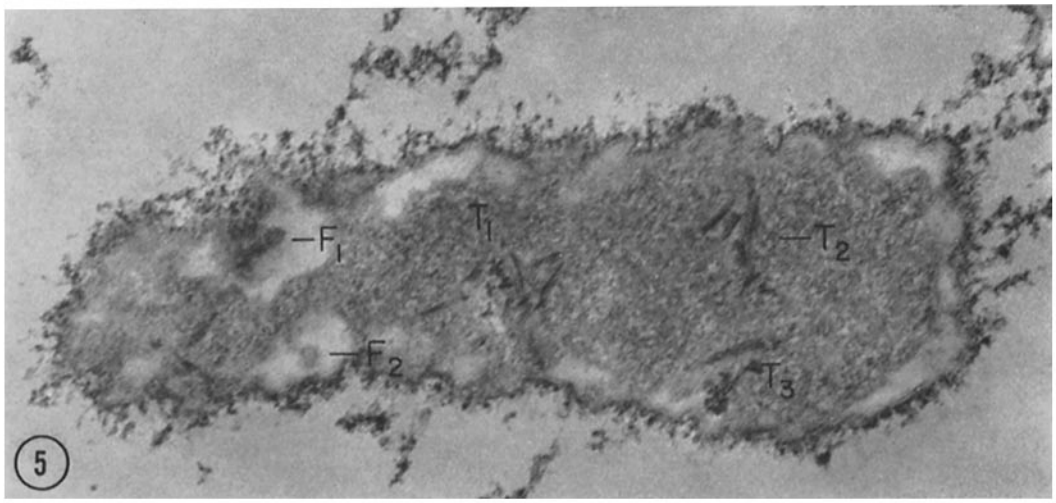
3


R
T2
T1
4


F1
T1
T2
F2
T3
5

of fine detail, it is clear from the cells in Figs. 16 and 17 that the deposits of the reduced tellurite are situated at the cell periphery, and from the cell in Fig. 16, which received a postfixation treatment with uranyl acetate, it can be learned that these deposits are apposed to the plasma membrane (arrows). Because, apart from the reduced tellurite, no heavy metals have been introduced into the cell shown in Fig. 17, the natural distribution of densities has been preserved here as much as possible.

DISCUSSION

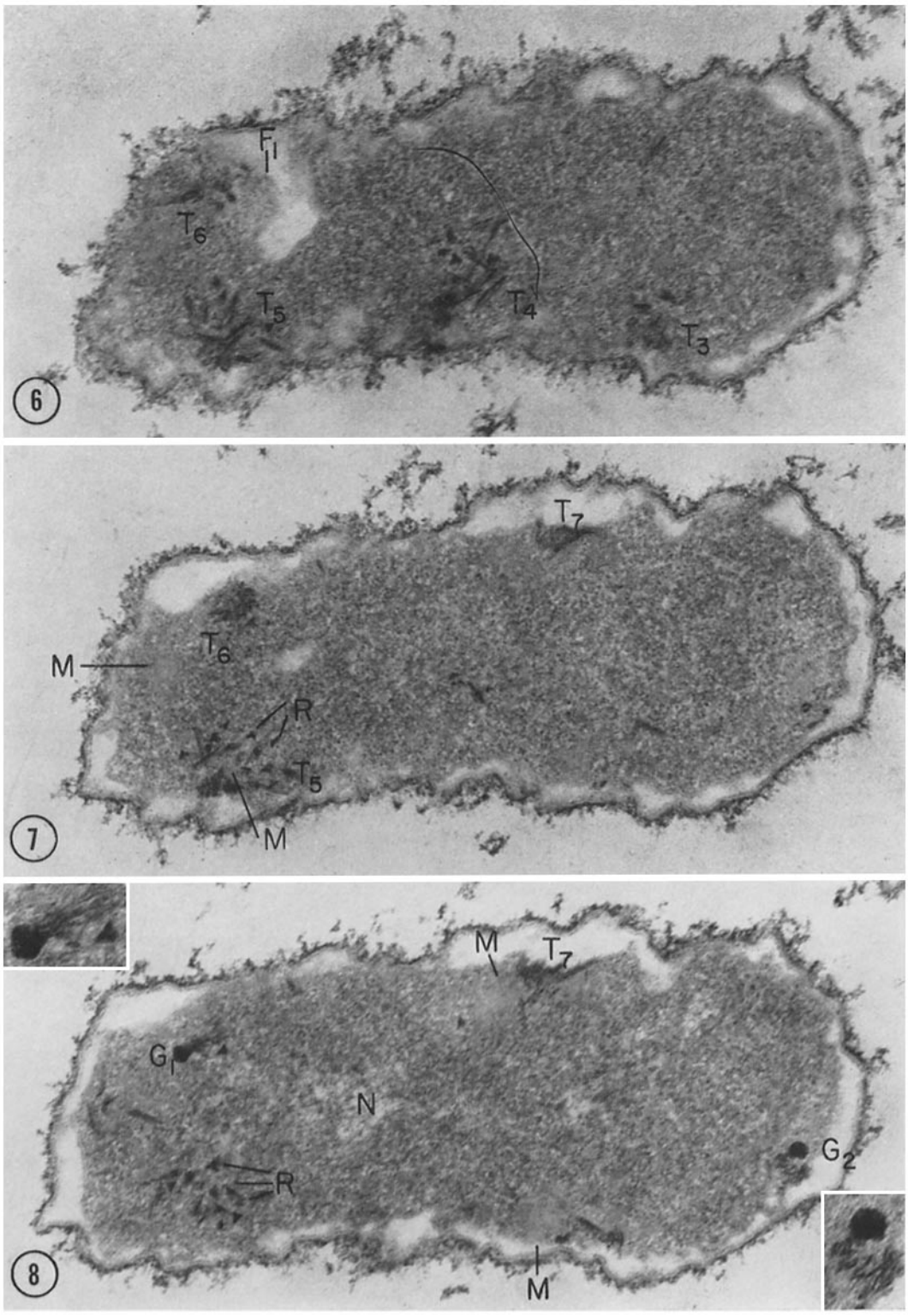
Several authors, of whom may be mentioned Wachstein (10), Mudd, Takeya, and Henderson (11), Terai, Kamahora, and Yamamura (12), and A. Winkler (13), have described inclusions of reduced tellurite for various species of bacteria. Thorough studies on the reduction of tellurite by *Proteus vulgaris* have been published more recently by Nermut (8), who stated that this reduction is of an enzymatic nature, the chemical explanation of which is unknown. Although not quite in accordance with Nermut's findings, Terai and co-workers (12) have also concluded that, in the bacterial reduction of tellurite, enzymes cooperate, one of which the authors have called tellurite reductase. Barrnett and Palade (14), who described fine "amorphous" deposits and relatively large "crystals" of reduced tellurite inside mitochondria of animal tissues, admit that the exact chemical nature of the final product is unknown. According to these authors, this product could be either tellurium oxide (TeO) or tellurium (Te), or both. Tucker and co-workers (15), using a procedure by which the bacteria could not be preserved in an intact healthy state, obtained a pattern of metallic tellurium by x-ray diffraction analysis. However, with the 8-hour-old cells dried on the supporting film, we were unable to obtain an electron diffraction pattern indicative of the presence of metal or metal oxide. Therefore, we are tempted to assume that in healthy living bacteria the reduced tellurite is present in, or on, organic structures. This idea is supported by our finding of structural details in the dense elements of the complexes. Demonstration of these organic structures in sections of untreated bacteria by means of staining is hardly possible because they are fairly thin in relation to section thickness and because the surrounding cytoplasmic particles are densely packed.

Dense "granules" in *Proteus vulgaris* treated with potassium tellurite have been convincingly demonstrated by Nermut (9) with the aid of the light microscope. As to the detailed structural analysis of these granules, this author was limited by the resolving power of the instrument. Consequently, his conclusion was restricted to the statement that the potassium tellurite is reduced either directly in the plasma membrane or in its immediate vicinity, probably in lipoid particles. Considering the impact of Weibull's well known work on the ghost of *Bacillus megaterium* (16, 17), it should be emphasized that in the present study the plasma membrane did not materially gain in electron opacity, but its contiguous particles incorporated the reduced tellurite. Our electron microscopic observations reveal a complex structure of the reducing "granules." These complexes appear to consist of a very fine granular "matrix" of low electron opacity, dense rod-like structures, and a mass of seemingly entangled and very delicate filamentous or lamellar structures, partly apposed

FIGURE 6 At F_1 the continuation of the same tube as that shown in the previous section. At T_3, T_4, T_5, and T_6 are conglomerates of elements which incorporated reduced tellurite.

FIGURE 7 At T_5, T_6, and T_7 are conglomerates of dense elements of which T_5 and T_6 are also visible in the previous section, and T_5, T_6, and T_7 in the next. The triangular structures at T_5 must be rods in cross-section. At M, a finely granular "matrix." At T_7 a dense mass is seen apposed to the plasma membrane (*cf.* T_1 and T_2 in Fig. 4 and the mass in Fig. 13).

FIGURE 8 At M, a finely granular "matrix," at R, triangular cross-sections of rods corresponding with site T_5, at G_1 a globular body corresponding with site T_6. The insets show the rounded structures G_1 and G_2 with delicate fibers or sheets attached to them. At N, the periphery of the nucleoplasm. Insets, × 158,000.


F1
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T4
T3
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G1
N
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G2
M
8

to the plasma membrane. The granular "matrix" described as a rather homogeneous body has been noticed for *Escherichia coli* (4, 18) and has been related to the site of tetrazolium reduction (19, 5). Shadowed specimens conveyed the impression that the flagella emerge from these tellurite-incorporating complexes (Figs. 1, 2), and we wonder whether they are the same as the basal granules formerly observed in autolyzed *Proteus* (20, 21).

The peripheral dense structures observed in *Bacillus subtilis* appear to be different in nature from those in *Proteus*, although there are indications that both serve the insertion of flagella. The dense rods in *Bacillus* are, in the sections, comparatively simple structures and may well represent the basal parts of the flagella, whereas the dense "granules" of *Proteus* are structurally complex. The Gram-negative bacteria like *Proteus* appear to lack the complex membranous organelles described for Gram-positive bacteria like *B. subtilis*. We wonder, therefore, whether the tellurium-incorporating complexes in *Proteus* may possibly combine two properties: being the bases of the flagella, and being the analogues of the membranous organelles. For the membranous organelles, evidence has been adduced that they are the mitochondrial equivalents in Gram-positive bacteria (2). Since no other sites of tellurite reduction were found in *Proteus vulgaris*, we suppose that the complex granules represent the mitochondrial equivalents or chondrioids (22) in this organism.

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FIGURES 9 to 13 Five serial sections of part of a conglomerate of the elements containing the reduced tellurite. × 126,000.

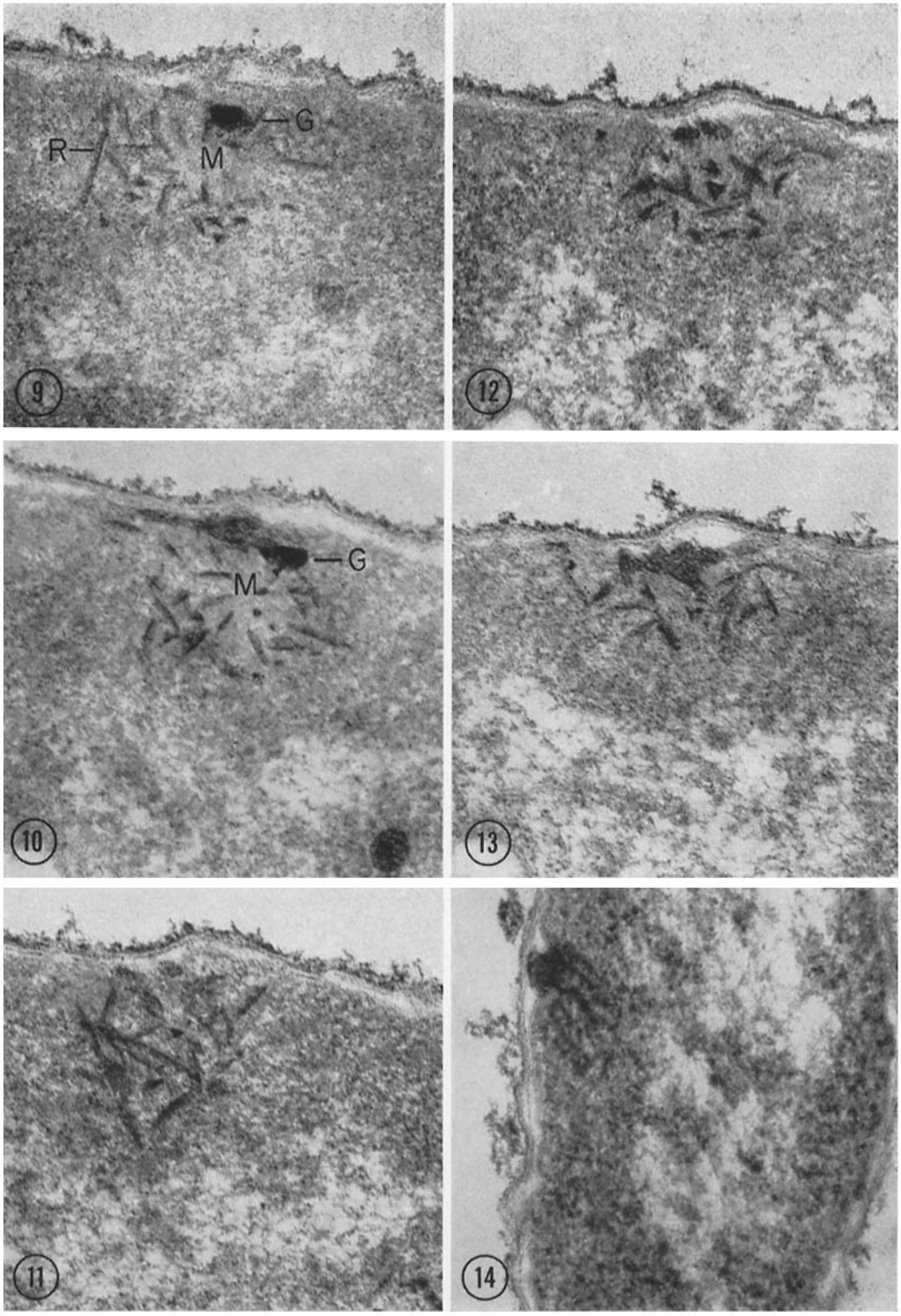
FIGURE 9 Finely granular "matrix" (*M*) present between rods. At *R*, indication in the rod of a periodic fine structure. At *G*, a rounded structure, visible also in the next section.

FIGURE 10 Conglomerate of rods around "matrix," and a rounded structure at *G*.

FIGURES 11 and 12 Rod-like structures.

FIGURE 13 This micrograph shows a dense mass apposed to the plasma membrane, comparable presumably to T_1 and T_2 in Fig. 4 and to T_7 in Figs. 7 and 8. Furthermore, the rod-like structures are present.

FIGURE 14 The reduced tellurite appears incorporated in very delicate lamellae. × 100,000.


R
G
M
9
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10
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11
14

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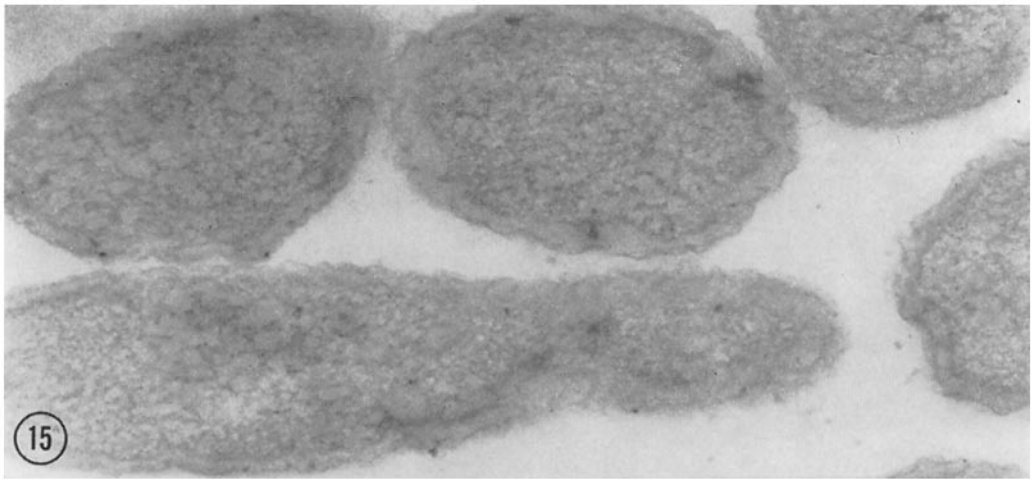
FIGURES 15 to 17 Sections of cells fixed with 6½ per cent glutaraldehyde instead of with OsO_4. Glutaraldehyde fixation resulted in a loss of structural detail but was applied in order to avoid the introduction of osmium.

FIGURE 15 Cell from control culture without potassium tellurite. The preparation has been given a postfixation treatment with uranyl acetate which has raised the contrast in the various cell structures. × 63,000.

FIGURE 16 Comparatively thick section showing clearly the conglomerates of elements in which the reduced tellurite has been incorporated, but the fixation applied preserved poorly their fine structure, even after posttreatment with uranyl. At arrows, the complexes are seen to be contiguous with the plasma membrane. × 63,000.

FIGURE 17 In order to preserve as much as possible a natural distribution of densities in the section, in this preparation the treatment with uranyl acetate was also omitted. The contrast between the cell and the Vestopal is very poor, but the reduced tellurite incorporated is well demonstrated at the periphery of the cell and at the location at which constriction takes place in preparation for cell division. × 63,000.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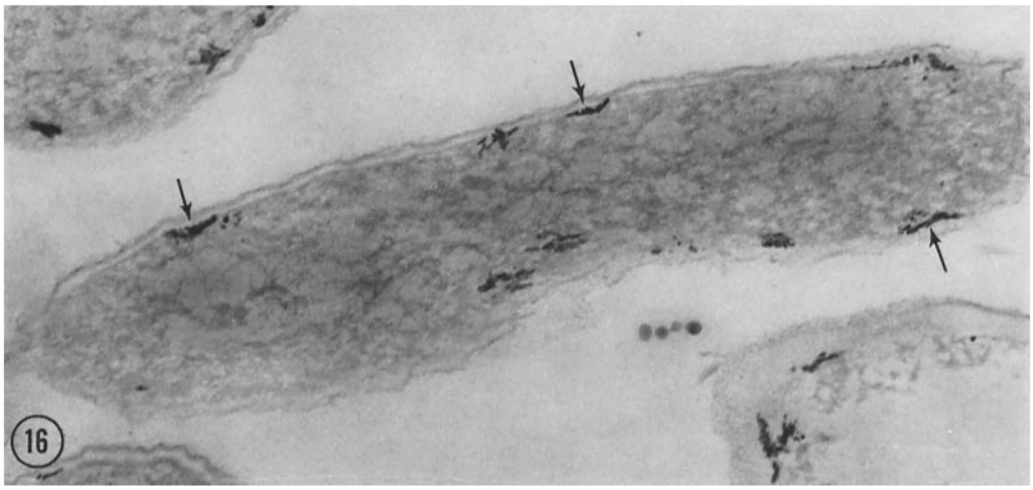
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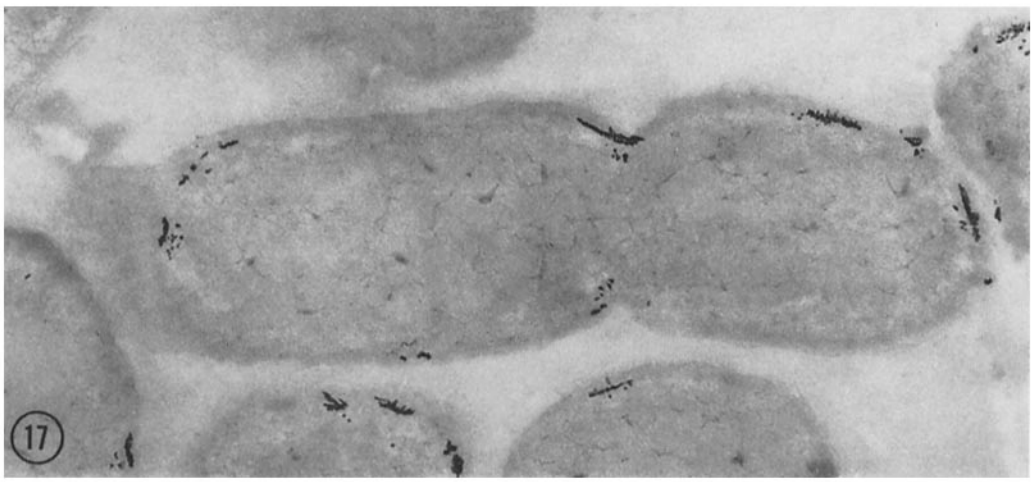
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